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Specific Detection of Echoviruses 22 and 23 in Cell Culture Supernatants by RT-PCR

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Reverse transcription–polymerase chain reaction (RT-PCR) methods are available for the rapid detection of enteroviruses in clinical specimens or virus isolates. Pan-enterovirus PCR primers, however, fail to amplify echovirus (E) type 22 or 23 because of their extreme sequence divergence from the other enteroviruses. We have developed an RT-PCR method to detect specifically E22 and E23 RNA directly in tissue culture supernatants without a viral RNA purification step. The E22/E23 primers successfully amplified 20 of 20 clinical isolates of E22 and 4 of 4 E23 isolates representing viruses isolated in 15 states over a 19-year period, as well as E22 and E23 prototype strains isolated in the 1950s. The primers did not amplify any of the other 64 enterovirus prototype strains. ***J. Med. Virol. 58: 178–181, 1999.*** Published 1999 Wiley-Liss, Inc.†

KEY WORDS: RT-PCR; echovirus 22; echovirus 23; molecular detection

INTRODUCTION

Nonpolio enteroviruses are common human pathogens, accounting for 30,000 to 50,000 pediatric hospitalizations for aseptic meningitis annually in the United States, in addition to causing other diseases such as encephalitis and myocarditis. Routine laboratory diagnosis of enterovirus infection is typically accomplished by virus isolation and neutralization with virus-specific antisera [Melnick et al., 1973]. This method is sensitive and specific, but highly labor-intensive. Definitive diagnosis may take several weeks because of the growth properties of the viruses and the need to titrate viral isolates carefully prior to the neutralization assay. In addition, the neutralization test may not be able to identify correctly antigenic variants or isolates that contain mixtures of two or more serotypes.

Reverse transcriptase–polymerase chain reaction (RT-PCR) has been used in many laboratories to detect enteroviruses in clinical specimens and in cell culture [Rotbart and Romero, 1995]. These methods target the 5′ nontranslated region (5′ NTR), which is highly conserved among all enteroviruses, except echovirus (E) types 22 and 23. E22 and E23 were originally classified as members of the genus *Enterovirus* (family *Picornaviridae*) based on classic viral taxonomic characteristics [Wigand and Sabin, 1961]. They possess many properties, however, which distinguish them from other enteroviruses, including distinctive cytopathology and a failure to shut off host protein synthesis [Shaver et al., 1961; Wigand and Sabin, 1961; Coller et al., 1990], and it has been proposed that they be reclassified in a separate genus within the *Picornaviridae* [Coller et al., 1991; Hyypiä et al., 1992; Stanway et al., 1994]. Of particular importance for molecular diagnostic development, the nucleotide sequences of E22 and E23 are highly diverged from those of conventional enteroviruses [Stanway et al., 1994; Oberste et al., 1998]. Published PCR methods for the detection of E22 and E23 failed to present data on the amplification of E22 and E23 prototype strains or clinical isolates, nor did they present data on the sensitivity of their systems, instead concentrating on other viruses detected during their studies [Shimizu et al., 1995; Read et al., 1997]. In addition, their methods required RNA purification prior to reverse transcription and PCR, adding an extra step to specimen processing and introducing an additional opportunity for specimen cross-contamination. Read et al. [1997] used a nested PCR protocol, potentially increasing sensitivity but also increasing the opportunity for contamination and false-positive results. The failure of pan-enterovirus RT-PCR methods to detect E22 and E23 [Hyypiä et al., 1989; Chapman et al., 1990; Zoll et al., 1992; Halonen et al., 1995] led us to develop a rapid RT-PCR method to detect E22 and E23 in virus cultures without purification of viral RNA.

MATERIALS AND METHODS

Viruses and RNA Extraction

The enterovirus prototypes and E22 and 23 strains used in the RT-PCR assays are shown in Tables I and

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TABLE I. Enteroviruses Used in RT-PCR Assays[a]

Virus serotype	Strain	Passage
CA1	Tompkins	SM9+
CA2	Fleetwood	SM10+
CA3	Olson	SM8+
CA4	High Point	SM1+
CA5	Swartz	SM9+
CA6	Gdula	SM9+
CA7	AB-IV	RD2
CA8	Donovan	SM10+
CA9	Griggs	MK3
CA10	Kowalik	SM8+
CA11	Belgium-1	SM9+
CA12	Texas-12	SM7+
CA13	Flores	SM9+
CA14	G-14	SM9+
CA15	G-9	SM1
CA16	G-10	HLF4
CA17	G12	SM9+
CA18	G-13	SM8+
CA19	8663	SM9+
CA20	IH-35	SM10+
CA21	Kuykendall	RD4
CA22	Chulman	SM8+
CA24	Joseph	SM10+
CB1	Conn-5	MK15+
CB2	Ohio-1	MK12+, BGM3
CB3	Nancy	HLF2, MK2, LLC-$MK_2$1
CB4	JVB	MK4, LLC-$MK_2$1
CB5	Faulkner	MK14, LLC-$MK_2$1
CB6	Schmitt	MK12+
E1	Farouk	MK2+
E2	Cornelis	MK15+
E3	Morrisey	MK14+
E4	Pesacek	MK13+
E5	Noyce	MK14+
E6	D'Amori	MK19+
E6′	Cox	MK6+
E6″	Burgess	LLC-$MK_2$5+
E7	Wallace	MK18+
E8	Bryson	MK19+
E9	Hill	MK20+, LLC-$MK_2$1
E11	Gregory	MK 12+, LLC-$MK_2$1
E12	Travis	MK14+
E13	Del Carmen	MK15+
E14	Tow	MK15+
E15	CH96-51	MK13+
E16	Harrington	MK18+, MRC5 2
E17	CHHE-29	MK17+
E18	Metcalf	MK18+
E19	Burke	MK20+
E20	JV-1	MK2
E21	Farina	MK18+, RU-1 2, HLF3
E22	Harris	MK3
E23	Williamson	MK1, BGM8
E24	DeCamp	MK18+
E25	JV-4	MK13+
E26	Coronel	MK13+
E27	Bacon	MK11+
E29	JV-10	MK9+
E30	Bastianni	MK13+
E31	Caldwell	MK6+
E32	PR-10	MK6+
E33	Toluca-3	MK16+
E34	DN-19	FL7+
EV68	Fermon	MK6, HFD7, HLF3
EV69	Toluca-1	HAM1, WI-38 3, HLF3
EV70	J670/71	HLF8
EV71	BrCr	RD1
PV1	Brunhilde	MK5
PV2	Lansing	MK4+, RD1
PV3	Leon	MK5+

[a]BGM, buffalo green monkey kidney cell; FL, fetal lung; MK, primary monkey kidney cell; RD, human rhabdomyosarcoma cell line, ATCC CCL 136; HLF, human lung fibroblast cell, ATCC CCL 199; SM, suckling mouse brain; WI-38, human diploid lung cell, ATCC CCL 75; LLC-MK_2; rhesus monkey kidney cell, ATCC CCL 7; MRC5, human diploid lung cell, ATCC CCL 171; RU-1, human foreskin fibroblast cell; HAM, human amniotic membrane cell; HFD, human diploid fibroblast cell.

II, respectively. In most cases, clarified cell culture supernatant was used directly in the RT-PCR assay, but for some experiments, RNA was extracted from positive control viruses by using sodium dodecyl sulfate and phenol [Oberste et al., 1998].

RT-PCR

RT and PCR steps were performed in a single tube containing 67-mM Tris-HCl, pH 8.8, 2-mM $MgCl_2$, 17-mM $(NH_4)_2SO_4$, 10-mM 2-mercaptoethanol, 6-μM EDTA, 0.2-mg/ml gelatin, 200 μM each dNTP, 0.1 μM each primer, 2-μl tissue culture supernatant, 4-U placental RNase inhibitor, 1.7-U AMV reverse transcriptase, and 1.3-U Taq DNA polymerase, in a total volume of 50 μl. Two different sense primers, K28 (AGCCATCCTCTAGTAAGTTTG, nucleotides 318–338) and K29 (TCTGGTAACAGATGCCTCTGG, nucleotides 417–437), were paired individually with antisense primer K30 (GGTACCTTCTGGGCATCCTTC, nucleotides 577–556). Prior to addition of enzymes, the reaction cocktail was incubated at 95C for 5 min to release RNA from intact virus. Enzymes were added and first-strand cDNA was synthesized for 30 min at 42°C, followed by 3 min at 95°C, and 35 amplification cycles of 95°C for 45 sec, 48°C for 45 sec, and 60°C for 45 sec. Control pan-enterovirus RT-PCR was performed as described [Yang et al., 1992]. Reaction products (15 μl each) were visualized by ethidium bromide staining and UV transillumination following electrophoretic separation of products in 12% polyacrylamide gels.

RESULTS

PCR primers specific for E22 and E23 were chosen on the basis of the published sequences of E22 [Hyypiä et al., 1992] and E23 [Oberste et al., 1998]. Primers were chosen from three regions within the 5′ NTR, which are highly conserved among E22 and E23 [Oberste et al., 1998]. Two different sense primers, K28 and K29, were chosen, and each was paired with an antisense primer, K30. Optimum primer concentrations and template sensitivity were determined by testing three primer concentrations, 0.1, 0.2, and 0.4 μM, with each of five template concentrations, 0.05 pg to 500 pg per reaction, using purified E23 RNA as template. For both primer pairs, a primer concentration of 0.1 μM produced a detectable product with no nonspecific products, while

TABLE II. E22 and E23 Strains for Testing of the E22/E23 RT-PCR Method

Specimen number	State	Date	Serotype
74-6099	CO	3/1/1974	E22
75-7039	CO	6/5/1975	E22
76-7535	TN	1/29/1976	E22
77-8640	AZ	4/3/1977	E22
79-1243	GA	11/20/1979	E22
81-2246	NC	2/3/1981	E22
81-2755	MA	9/21/1981	E22 and E23
82-3193	MI	2/17/1982	E22
82-3198	MD	2/24/1982	E22
82-3964	PA	9/27/1982	E22
82-3980	MA	9/30/1982	E22
83-4496	NC	4/21/1983	E22
85-6033	MT	6/19/1985	E23
86-6763	OK	11/21/1986	E22
86-6764	NM	12/3/1986	E22
88-8143	HI	1988	E22
88-8144	HI	5/13/1988	E22
88-8218	MD	6/29/1988	E22
89-8783	MN	1/27/1989	E22
89-9713	OK	12/13/1989	E22
91-0546	MD	3/15/1991	E23
91-1427	OR	11/26/1991	E23
92-1451	MO	3/25/1992	E22

higher primer concentrations resulted in the synthesis of nonspecific reaction products (data not shown).

Both primer pairs, K28/K30 and K29/K30, specifically amplified both E22 and E23, producing the expected products of 260 bp and 161 bp, respectively, using 2 μl of cell culture supernatant as template (Fig. 1). Neither primer pair produced a product when any other enterovirus was used as template (Fig. 1). In contrast, pan-enterovirus primers EV1/EV2 produced a product of 114 bp from every enterovirus template except from E22 and E23 (data not shown), confirming the presence of amplifiable template in the K28/K30 and K29/K30 reactions shown in Figure 1.

To determine whether the E22/E23-specific RT-PCR primers could be expected to amplify all clinical isolates of E22 and E23 encountered in a diagnostic laboratory, we tested the primers against a panel of E22 and E23 isolates received in our laboratory for reference diagnostic services between 1974 and 1992 (Table II). Each of the viruses had been typed using standard Lim Benyesh-Melnick antiserum pools [Melnick et al., 1973] and type-specific antisera as part of routine reference diagnostic procedures (data not shown). The virus panel contained 20 E22 strains from 13 different U.S. states, isolated over a 19-year time period, and 4 E23 strains isolated between 1981 and 1991, each from a different state. Both primer pairs amplified the expected product from each of the E22 and E23 virus isolates (Fig. 2), while the pan-enterovirus primers failed to amplify any product from any of the E22 or E23 isolates (data not shown).

DISCUSSION

The gold standard in enterovirus identification from clinical specimens is the neutralization assay, using pooled or individual antisera of defined reactivity [Melnick et al., 1973]. This method works well, but is time-consuming and very labor-intensive, and the required serologic reagents are in finite supply and expensive to produce. Because of the shortcomings of classic serologic typing, several laboratories have developed molecular methods to detect enteroviruses by using probe hybridization or RT-PCR [Rotbart and Romero, 1995]. The molecular methods have the advantage that the required reagents can be propagated (plasmid probes) or synthesized (oligonucleotide primers and probes) simply and cheaply. PCR techniques can be used to detect enteroviruses in a clinical specimen or a virus isolate with high sensitivity, but current methods do not permit the identification of the individual enterovirus serotype. Viruses identified as enteroviruses still must be serotyped by the classic methods.

Using cell culture to separate cultivable from noncultivable enteroviruses and, coupled with available pan-EV and pan-polio PCR primers [Yang et al., 1992; Kilpatrick et al., 1996], our E22/E23 RT-PCR method is an additional step toward molecular typing of enteroviruses by RT-PCR, allowing classification of an enterovirus isolate as "polio," "E22/E23," or "other cultivable enterovirus." Our method takes advantage of the fact that E22 and E23, while similar to other enteroviruses in their disease spectrum, are distinct in nucleotide sequence yet remaining very similar to one another [Hyypiä et al., 1992; Stanway et al., 1994; Oberste et al., 1998]. This assay detected 21 of 21 E22 isolates (the prototype strain plus 20 clinical isolates) and 5 of 5 E23 isolates (the prototype strain plus 4 clinical isolates), representing a broad temporal and geographic sampling of available strains, with no amplification of non-E22/E23 enterovirus prototypes.

Read et al. [1997] have developed a nested RT-PCR method to amplify E22 from purified RNA derived from cerebrospinal fluid; however, each of their outer PCR primers contains five mismatches with the homologous sequence of E23 and would therefore be unlikely to amplify E23. In addition, they presented no data on the specificity of their method, nor did they detect E22 from any of their clinical specimens. The primers of Shimizu et al. [1995] were very similar to K28 and K30 and would be expected to detect both E22 and E23, based on the complete conservation of sequences at the annealing sites. Like Read et al. [1997], Shimizu et al. [1995] failed to show specificity data and they also did not detect either E22 or E23 from clinical specimens in their study. Neither of the published E22/E23 PCR methods demonstrated amplification of recent clinical isolates, calling into question the ability of their techniques to detect currently circulating strains, in light of the high rate of sequence evolution among the picornaviruses. Since there is currently no other molecular method available to differentiate rapidly contemporary nonpolio enteroviruses, our E22/E23 RT-PCR provides a means to distinguish E22 and E23 from all other cultivable enteroviruses. Coupled with continued development of PCR methods that can distinguish among

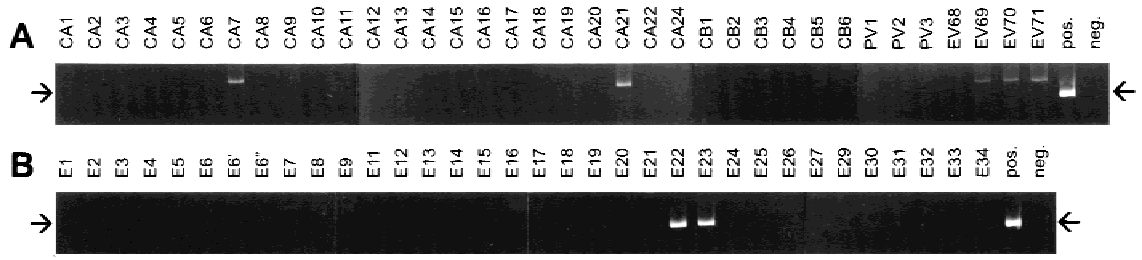

Fig. 1. Amplification of enterovirus prototype strains with primer pair K28/K29. Arrows indicate the position of the expected 260-bp product. Identical results were obtained using primer pair K29/K30 (data not shown). Pos., positive control (E23 RNA); neg., negative control (no template). **A**: Amplification of coxsackie A viruses (CA), coxsackie B viruses (CB), polioviruses (PV), and numbered enteroviruses (EV), **B**: Amplification of echoviruses (E).

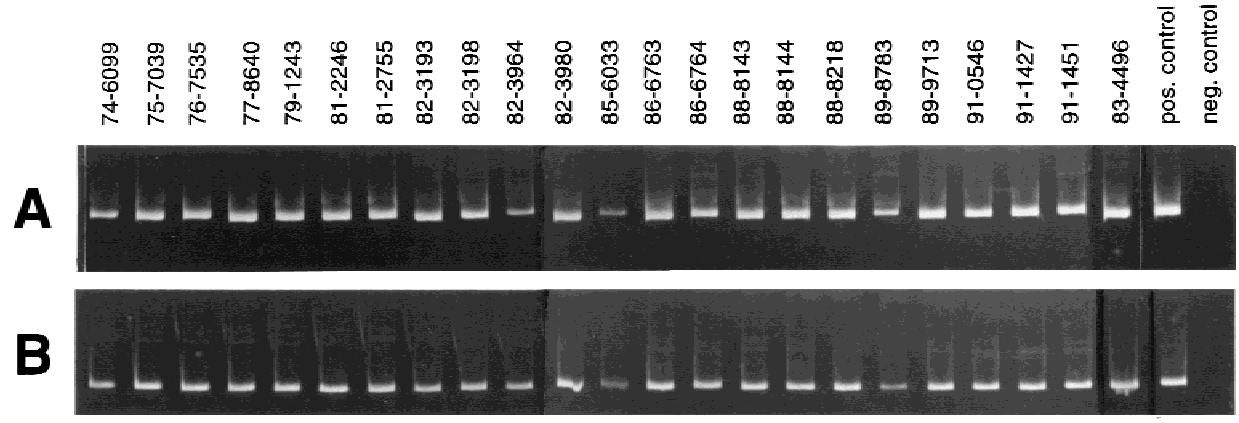

Fig. 2. Amplification of clinical isolates of E22 and E23 using primer pairs K28/K30 and K29/K30. The isolate identifier is indicated above each lane. Pos., positive control (E23 RNA); neg., negative control (no template). **A:** Amplification with primers K28/K30. **B:** Amplification with primers K29/K30.

other enterovirus groups, our E22/E23 method will allow PCR to be used for both initial screening and typing of clinical isolates, with neutralization used as a confirmatory test, thus preserving valuable serologic reagents and reducing the long-term cost of enterovirus laboratory diagnosis.

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